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(54) Title: ASSAY METHOD

(57) Abstract: A method for the identification of a compound having bone growth promoting activity comprising contacting a test compound with a biological system which comprises a functional RORα receptor and which is capable of generating a detectable signal in response to activation of the RORα receptor by a compound having bone growth promoting activity, and monitoring the system for the generation of the signal and the use of such compounds for the prophylaxis or treatment of diseases or medical conditions which involve excessive bone or cartilage loss.

ASSAY METHOD

This invention relates to biologically active organic compounds, in particular to compounds which have activity as promoters of bone growth and development and to processes for the identification of such compounds and also to the therapeutic use of such compounds.

There are a number of diseases and medical conditions which involve excessive bone or cartilage loss, including osteoporosis, gingival disease such as gingivitis and periodontitis, Paget's disease, tumour induced hypercalcemia and metabolic bone disease. Compounds which have activity as promoters of bone growth and development are potentially useful for the therapeutic treatment of such diseases and conditions.

RORα (retinoic acid related orphan receptor alpha, also known as RZR) was cloned and functionally characterised by Giguère et al. (GENES & DEVELOPMENT 8:538-553, 1994) as a new member of the steroid hormone nuclear receptor super family, with an unknown ligand binding specificity or physiological role. RORα has three isoforms, referred to as RORα1, RORα2 and RORα3, of which the RORα1 and RORα2 isotypes were found to bind as monomers to hormone response elements present in certain gene sequences and composed of an AT rich sequence preceding a half site core motif PuGGTCA. Recently it has been reported (Wiesenberg et al. Nucleic Acids Research, 1995, Vol.23, No.3 327-333; Steinhilber et al. J. Biol. Chem. 1995, Vol. 270, No. 13, 7073-7040) that RORα1 is a nuclear receptor for the pineal gland hormone melatonin.

We have now found, in accordance with the present invention, that expression of RORα mRNA is differentially upregulated during the differentiation of mesenchymal stem cells into osteoblasts, indicating that RORα is involved in the development and differentiation of bone-forming cells. Furthermore we have shown that the mouse mutant staggerer (sg), which has a deletion within the RORα gene which prevents translation of the RORα ligand binding domain, exhibits an osteopenic phenotype, indicating that expression of fully functional RORα is required for a normal, healthy

bone phenotype. Furthermore animals which are heterozygous for the deletion exhibit accelerated bone loss with aging. Taken together these results support the proposition that activation of RORα is a key step in the activation of expression of bone related proteins by RORα and in bone growth and development. Thus, in accordance with the present invention, RORα is proposed as a new therapeutic target for treatment of diseases and medical conditions which involve excessive bone or cartilage loss, such as osteoporosis.

Accordingly in a first aspect the invention provides a method for the prophylaxis or treatment of a disease or medical condition which involves excessive bone or cartilage loss comprising administering to a patient an effective amount of an activator of RORα.

In an alternative embodiment of the first aspect the invention provides the use of an activator of RORα in the preparation of a medicament for the treatment of a disease or medical condition which involves excessive bone or cartilage loss.

The diseases or medical conditions which involve excessive bone or cartilage loss which may be treated according to the invention include all bone conditions which are associated with increased calcium depletion or resorption or in which calcium fixation in the bone is desired, e.g. osteoporosis of various genesis (e.g. juvenile, menopausal, postmenopausal, post-traumatic, post-transplantation, caused by old age, by cortico-steroid therapy or by inactivity), fractures, osteopathy, including acute and chronic states associated with skeletal demineralisation, osteo-malacia, gingival disease such as gingivitis and periodontitis, Paget's disease, tumour induced hypercalcemia, metabolic bone disease, or bone loss due to arthritis or osteoarthritis. In particular the invention may be used for the treatment or prophylaxis of osteoporosis of various genesis.

In particular the invention relates to the use of activators of RORα1 to treat diseases of excessive bone or cartilage loss.

The activators of RORα which may be used in the present invention are preferably compounds not previously known to be activators of RORα, including novel compounds.

International patent application WO 95/27202 describes use of a receptor of the RZR/ROR receptor family or a functional fragment thereof in a test for identifying a compound with anti-autoimmune, anti-arthritic, anti-tumour, melatonin like and/or melatonin antagonist activity. The testing procedures described in WO 95/27202 may be used to identify compounds which have bone growth or development promoting activity. The disclosure of WO 95/27202, in particular the disclosure relating to to the use of a receptor of the RZR/ROR receptor family or a functional fragment thereof in a test for identification of a compound RORα, e.g. RORα1 activating activity, is incorporated by reference into the teaching of the present application.

Thus in a further aspect the invention provides use of a receptor of the RZR/ROR receptor family, e.g. RORα, especially RORα1, or a funtional fragment thereof in a test for identifying a compound with bone growth or development promoting activity.

In a preferred embodiment of this further aspect the invention provides a method for the identification of a compound having bone growth or development promoting activity which comprises contacting a test compound with a biological system which comprises a functional RORα receptor and which is capable of generating a detectable signal in response to activation of the RORα receptor by a compound having bone growth or bone formation promoting activity, and monitoring the system for the generation of the signal.

Thus for example, advantage can be taken of the known interaction between the RORα ligand binding domain (RORα-LBD) and the coactivator GRIP. This interaction will be modified by the binding of a ligand to the RORα-LBD and induce a change of conformation which translates into differences in fluorescence which can be monitored using standard techniques

The method of the invention may be used to screen individual compounds and libraries of compounds, including combinatorial compound libraries. The method may be used as a first line screening assay to identify lead compounds and may be used to compare or quantify the RORα activating activity of compounds, e.g. to compare compounds produced from medicinal chemistry lead optimisation/derivatisation programmes.

Thus in preferred embodiments the invention provides:

i) a method for the identification of a compound having bone growth or formation promoting activity comprising contacting the compound with a biological system which comprises a functional RORα receptor and which is capable of generating a detectable signal in response to activation of the RORα receptor by a compound having bone growth promoting activity, measuring the detectable signal in the presence of the test compound and comparing the result obtained with a control, and

ii) a method for the comparison of compounds which have bone growth or formation promoting activity, comprising separately contacting the compounds with a biological system which comprises a functional RORα receptor and which is capable of generating a detectable signal in response to activation of the RORα receptor by a compound having bone growth promoting activity, measuring the detectable signal in the presence of each test compound and comparing the signals obtained.

Suitable procedures for carrying out the identification and comparison methods of the invention include those described in WO 95/27202

In the present description "bone growth promoting activity" means increased proliferation of osteoblast precursors cells, increased differentiation of such cells, increased matrix synthesis by osteoblasts, prolonged life span of osteoblasts or osteocytes, for instance, leading ultimately to increased bone mass or maintenance of bone mass . The invention is further described by way of illustration of the invention only in the following examples which relates to a particular assay of the invention and refer to the accompanying figures:

Figure 1, which shows agarose gel electrophoresis patterns of PCR products obtained from mRNA samples collected at different time of human mesenchymal stem cells differentiation.

Figure 2, which is a graph showing Quantitative analysis of the expression of RORα during human MSCs differentiation ;

Figure 3, which is a graph showing induction of mouse bone sialoprotein promoter activity by RORα.

Figure 4, which is a graph showing RORα repression of the osteocalcin promoter is dominant over vitD activation.

Figure 5, which are graphs showing shows the bone mineral content, area, density, and length (d) of the whole tibia of mice with a deletion within the RORα gene copmpared to wildtype mice as evaluated by double energy X-ray absorptiometry (DEXA);

Figure 6, which are graphs showing the total bone mineral content, area, and density in a cross section of the proximal tibia of mice with a deletion within the RORα gene copmpared to wildtype mice as evaluated by peripheral quantitative computed tomography (pQCT);

Figure 7, which are graphs showing the cortical thickness and trabecular bone mineral density in a cross section of the proximal tibia of mice with a deletion within the RORα gene copmpared to wildtype mice as evaluated by peripheral quantitative computed tomography (pQCT), and

Figure 8, which is a graph showing the trabecular bone mineral density in a cross section of the proximal tibia of ageing mice which are heterozygous for a deletion within the RORα gene compared to ageing wildtype mice as evaluated by peripheral quantitative computed tomography (pQCT);

EXAMPLES

Example 1: Differential Expression of RORα during Differentiation of Mesenchymal Stem Cells /Analysis of the functional ability for RORα

Experimental procedures

Human Mesenchymal stem cell culture and RT-PCR. Human mesenchymal stem cells, derived from 4 different donors, were cultivated and differentiated (osteogenic pathway) according to standard protocols (1 μM dexamethasone; 50 μM ascorbic acid-2-phosphate; 100 μM β-glycerophosphate). The cells were harvested after 0, 4, 8, and 15 days after starting the treatment and processed according to the manufacturers protocol for total RNA preparation (RNeasy midi RNA, Quiagen). 100 ng of total RNA was added to PCR-reactions containing the appropriate primers for RORα (5'-3'forward primer, GTAGAAACCGCTGCCAACA, reverse primer, ATCACCTCCCGCTGCTT), and the reaction mix (Superscript one-step RT-PCR system, GibcoBRL). PCR conditions were: cDNA synthesis at 50°C, 30 min; 94°C, 2 min; 95°C, 1min; 56, 30 sec; 72°C, 1 min. All experiments were performed twice using RNA-preparation from MSCs derived from different donors. The PCR-fragments were visualized on a 1.5% agarose gel.

Quantitative real time PCR (Taqman assay). This technique was used to quantitatively monitor mRNA expression. mRNA was extracted from hMSCs or ROS17 2.8 cells cultured as described in other sections of experimental procedures. A gene-specific PCR oligonucleotide primer pair and an oligonucleotide probe labeled with a reporter fluorescent dye at the 5`-end and a quencher dye a the 3`-end were designed using primer express 1.o software. The primer and probes used were as follows: hRORα gene (5`-3`):

forward primer GTGCGACTTCATTTTCCTCCAT;

reverse primer GCTTAGGTGATAACATTTACCCATCA;

and probe CACTTCAGAATTTGAGCCAGCAATGCAA.

mRNA (10 ng) was added to a 50 µl RT-PCR reaction mix (Perkin Elmer). The thermal cycle conditions included 1 cycle at 50°C for 30 min, 1 Cycle at 95°C for 10 min alternating 40 cycles at 90°C for 15 sec, 40 cycles at 60°C for 1 min.

Preparation of promoter constructs and plasmids. The plasmid pBS 2.5 BSP (kind gift of J. Aubin, University of Toronto) containing the promoter region of the target gene was cut with XhoI and XbaI to obtain a 2.5 kb fragment of the mouse BSP promoter. The fragment was ligated into the XhoI and NheI sites of pGl2-basic vector (Promega, Wi) to drive the firefly luciferase gene (BSP-luc). The RORα1 expression construct and the DR8tk luc were obtained from Dr. M Becker-Andre, Geneva and are described previously (Giguère et al.,GENES & DEVELOPMENT 8:538-553, 1994). The osteocalcin promoter constructs are described in Meyer et al. (J Biol Chem., 272, 21090-21095, 1997).

Cell culture, transient transfections and luciferase assay.

ROS 17/2.8 and MG63 cells were cultered at 37°C in a humified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium/F12 nutrition mixture buffered with bicarbonate and supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). Cells were seeded in 6 well plates 24 hours before a transfection and transfected at 50-60% confluence using FuGene6 transfection reagent (Boehringer Mannheim). A typical reaction mixture contained 2 µg reporter plasmid and 1 µg expression plasmid. After 4 h exposure to the transfection mix, medium was refreshed and cells treated for 24 with Vit D3 if indicated. Transfected cells were subsequently harvested for luciferase assay by scraping the cells into in 0.25 ml lysis buffer (Promega, Madison, Wi) after washing them in phosphate-buffered saline. Luciferase activity was monitored according to the Promega luciferase assay kit using an automatic luminometer LB96P (Berthold, Regensburg, Germany). Results are expressed in relative light units (RLU) per mg protein. All experiments were performed in triplicate on three separate occasions.

Results

The experimental basis for this study relied on a reverse transcriptase (RT)-PCR coupled reaction and the use of RNA prepared from hMSC at different stages during osteogenic differentiation. We directly compared samples of cells in a treated (OS) or untreated stage. In the case of RORα, we monitored a differential expression pattern of distinct RORα messenger signals during the commitment of MSCs into the osteogenic pathway. The results obtained are given in Figure 1, which shows agarose gel electrophoresis patterns of PCR products obtained from mRNA samples collected at different time points during human MSC differentiation. The identity of the PCR product obtained in these experiments was assessed by subcloning and sequencing analysis. Other members of the ROR subfamily such as RORβ and RORγ were not detected in human MSCs. RNA prepared from cells of different origin (brain) were used as as a positive control (C).

In addition, we set up a real-time PCR technique, which allowed us to quantify the RORα expression at different stages of the osteoblastic differentiation. RORα expression was normalised with GAPDH. The results obtained are given in Figure 2, which is a graph of the quantitative determination of fold induction of RORα obtained by comparing RORα levels in samples of cells in a treated (OS) or untreated stage (C), related to the expression level of cells in the native state (day 0).

Next the functional importance of RORα was examined in cellular transfection assays using the rat osteosarcoma cell line 17/2.8 (ROS 17/2.8) as host cells. Given the background that secreted components of the bone organic matrix like bone sialoprotein (BSP) or osteocalcin (OC) are modulators of mineralization and remodeling a conditionally active transcription factor like RORα, which is believed to be involved in regulation of bone metabolism, should regulate the promoters of these bone specific genes. The BSP has already been suggested as a potential ROR target gene since Schrader et al.(JBC, 271, 19731-19736, 1996) identified already hexameric consensus motifs RGGTCA (R=A or G) within the human and rat BSP promoter sequence. Within the mouse BSP promoter we found similar consensus element located (GGGTCA) between position –2007 to-2001 in respect to the transcription start site. Therefore, a 2500 bp spanning fragment of the mouse BSP promoter was used to drive

the firefly luciferase gene. Ros17/2.8 cells were transfected with parent expression vector (Crt) or expression vector for RORα together with a luciferase reporter gene. The reporter gene was driven by the 2500 promoter fragment of the mouse bone-sialoprotein gene. Luciferase activity was assayed in cells from 6 well plates and related to the activity in cells transfected with an empty expression plasmid. The results were normalized to the protein content. The Figure 3 shows the mean +SD of three experiments, each carried out with three independent triplicate analyses. We used a RORα1 cDNA since the α2 or α3 version was not detectable by RT-PCR in hMSCs (data not shown) and furthermore, since it has been described that RORα1 has the strongest transcriptional activity of the three subtypes. Co-expression of RORα resulted in a 7 fold increase in luciferase activity of the BSP luc construct compared to basal levels as shown in Figure 3.

This increase in luciferase activity was similar to the one obtained with a reporter construct containing a simple RORα response element (DR8) under the same conditions (data not shown). Further in vivo evidence for RORα action in bone cells was collected by studying the effect of RORα on the osteocalcin gene activity. As shown in Fig. 4, ROS 17/2.8 cells were transfected with a luciferase reporter gene driven by either -344/+34 or – 890/+34 of the human osteocalcin promoter together with an expression vector for RORα (2/1 mg) (hatched bares) or without (solid bars). The cells were incubated with 10 nM vitD. Luciferase activity was assayed in cells from 6 well plates and related to the activity in cells transfected with an empty expression plasmid. The results were normalized to the protein content. The figure shows the mean +SD of three experiments, each carried out with three independent triplicate analyses. Overexpression of RORα had no influence on the basal activity of the OC-890 promoter spanning nucleotides –890/+34 or on a shorter promoter construct OC-344 spanning nucleotides –344/+34. As expected only the OC-800 promoter construct was regulated by vitamin D3 up to 5 fold, since a palindromic DNA sequence shown to bind the VDR/ retinoid X receptor (RXR) heterodimer is located between bp -513 –493 upstream of the transcription start site of the human osteocalcin promoter. The cotransfection of the RORα expression plasmid with the

OC-890 construct resulted in a partial suppression of the vitamin D-activated level, up to 50%, of the OC-890 promoter reporter gene as shown in Figure 4.

Summary

The results obtained show evidence for a differential expression of RORα during the differentiation of hMSCs into osteoblasts and support the proposition that RORα is involved in bone development. Moreover RORα is able to transcriptionaly regulate two major non collagenous proteins, bone sialoprotein and osteocalcin. In the case of the bone sialoprotein we observed a positive regulation, whereas in the case of osteocalcin we observed a repression of the vitamin D activated osteocalcin transcriptional level. This has two major implications. Osteocalcin itself is a major player in bone metabolism since it has been shown that in osteocalcin deficient mice bone formation was increased Ducy et al. (NATURE, 1996, 382:448-452). In addition vitamin D is a major calciotrophic hormone and we demonstrate a cross/talk between the vitamin D receptor and RORα. Taken together these data suggest a physiological role of RORα in bone cell differentiation and in regulation of important bone non collagenous protein.

Example 2: Screening Assay

ROR α expression is correlated with osteoblast differentiation. From in vitro and in vivo data we can conclude that RORα plays a central role in bone metabolism. Thus an activator of RORα may be potentially useful for the treatment of osteoporosis. The search for a ligand may be done in different ways. A cell line, which has stably integrated an expression vector for RORα and a reporter containing direct repeats of the sequence AGGTCA driven by a tk promoter linked to a luciferase reporter gene is used. Direct repeats have been shown to be binding site for RORα.

Plasmids used

The expression vector pCMX RORα1 has been described by Giguere et al.(Genes & Development, 8:538-553). The reporter plasmid consists of two repeats of the

sequence AGGTCA with an 8 bp spacing placed in front of a tk minimal promoter driving the firefly luciferase reporter gene.

Establishment of a stable cell line

The expression vector for RORα1 and the reporter (DR8)tk-luc are transfected in Ros17/2.8 cells using Fugene as described in the previous section. In addition, an antibiotic resistant gene construct such as the puromycin resistant gene is cotransfected with these two plasmids. Cells which have stably integrated the puromycin resistant gene as well as the reporter and the expression vector for RORα produce stable cell lines suitable for the search of RORα ligands or activators.

Example 3: Examination of the bone phenotype of Staggerer (sg) Mice

Animals

The mouse mutant staggerer (sg) – a mutation, which occurred spontaneously in a stock of obese mice in 1955 - maps within 160 kb of the position of RORα, suggesting the latter as the site of the sg mutation. A deletion within the RORα gene that prevents translation of the ligand-binding domain was found in staggerer mice. Homozygotes for the staggerer show a staggering gait, mild tremor, hypotonia, and small size. The cerebellar cortex is grossly underdeveloped with a deficiency of granule cells and Purkinje cells. RORα KO mice generated in the mid-nineties display a similar phenotype.

We collected bones from 16 week old homozygote (sg/sg), heterozygote (sg/+), and wildtype (+/+) male mice (n=10/group, littermates from several mothers) to determine whether the bone phenotype of these mice would be changed by the deletion within the RORα gene.

We also collected the bones from 10 and 18 month old heterozygote (sg/+), and wildtype (+/+) male mice to determine whether the bones of heterozygous mice would exhibit the same rate of bone loss with aging as those of wildtype mice.

Methods

The excised left tibia was put into 4°C fixative (Karnovsky) for 24 hours followed by dehydration. X-ray based evaluations were performed in 70% ethanol (DEXA, pQCT).

X-rays

X-rays (Mammomat, Siemens, Dietikon, Switzerland) were taken for measurement of bone length.

Dual-energy X-ray absorptiometry - DEXA

Tibial bone mineral content (BMC) and bone mineral density (BMD, mg/cm^2) were measured using a regular Hologic QDR-1000 instrument (Hologic, Waltham, MA, USA) adapted for measurements of small animals. A collimator with 0.9 cm diameter and an ultrahigh-resolution mode (line spacing 0.0254 cm, resolution: 0.0127 cm) was used. The stability of the measurement was controlled daily by scanning a phantom.

Peripheral quantitative computed tomography – pQCT

Cortical and cancellous bone mass and geometry were monitored in the proximal tibia metaphysis 2.5 mm distal to the medial and lateral intercondylar tubercle using a Stratec-Norland XCT-2000 (Pforzheim, Germany). The following setup was chosen for the measurements: voxel-size: 0.1 mm x 0.1 mm x 0.5mm (slice thickness), scan speed: scout view – 10 mm/s, CT – 2 mm/s, 1 block, contour mode 1, peelmode 2, cortical threshold: 400 mg/cm^3. The bones were placed into a plastic container filled with 70% ethanol. The stability of the measurement was controlled daily by scanning a phantom.

Statistical analysis

The results are expressed as mean ± standard error (SEM). All statistical analysis was carried out using BMDP (Version 1990 for VAX/VMS, BMDP Statistical Software Inc., Cork Ireland). The data were subjected to one-way analysis of variance (ANOVA). Levene F-test was used to test for equality of variances, and differences between groups were tested using Dunnett test (significance level: $p<0.05$). All statistical tests were two-tailed. Differences between all groups were tested for statistical significance.

Results

DEXA

As shown in Figure 5 the bone mineral content of the tibia was significantly reduced in homozygote sg/sg mice compared to heterozygote sg/+ and wildtype +/+ mice (Figure 5a). This was mainly due to a decreased density (Figure 5c) and not to reduced bone size (Figure 5b, d). (Figure 5: Bone mineral content (a), area (b), density (c), and length (d) of the tibia (DEXA); Mean ± SEM; ANOVA, Dunnett, $p<0.05$, a = sg/sg ⇔ +/+, b = sg/sg ⇔ sg/+;)

PQCT

The total bone mineral content of a cross section through the proximal tibial metaphysis was significantly reduced in the sg/sg animals compared to sg/+ and +/+ animals (Figure 6 a). This was both due to a reduced cross-sectional area (Figure 7 b), indicating a thinner tibia metaphysis in those animals, and to a decreased mineral density (Figure 6 c). Cortical thickness and trabecular density were also reduced (Figure 7 a, b). The heterozygotes (sg/+) did not display this bone phenotype at all. Compared to control wildtype animals (+/+) they showed similar bone geometry and mass (Figure 6, 7) at the age of 4 month, when the animals had reached their peak bone mass. However bone loss was accelerated with aging in heterozygous (sg/+) animals as indicated by an increased loss of cancellous bone density compared to wildtype (+/+) animals (Figure 8). In addition we found that the animals display a stiffness in their hindlimbs, suggesting accelerated joint degeneration.

(**Figure 6:** Total bone mineral content (a), area (b), and density (c) in a cross section of the proximal tibia (pQCT); Mean ± SEM; ANOVA, Dunnett, $p<0.05$, a = sg/sg ⇔ +/+, b = sg/sg ⇔ sg/+;)

(**Figure 7:** Cortical thickness (a) and trabecular bone mineral density (b) in a cross section of the proximal tibia (pQCT); Mean ± SEM; ANOVA, Dunnett, $p<0.05$, a = sg/sg ⇔ +/+, b = sg/sg ⇔ sg/+, c = sg/+ ⇔ +/+;)

(**Figure 8:** Trabecular bone mineral density in a cross section of the proximal tibia (pQCT); Mean ± SEM; ANOVA, Dunnett, $p<0.05$; c = sg/+ ⇔ +/+;)

Summary

The homozygote mouse mutant staggerer, who has a deletion within the RORa gene, has a reduced long bone diameter compared to heterozygotes and the wildtype. More interestingly the bones of the homozygote (sg/sg) animals are osteopenic as indicated by bone mineral measurements (DEXA, pQCT) compared to the wildtype (+/+) animals. Mice, who are heterozygous for the deletion (sg/+), develop normal bone mass. However they exhibit accelerated bone loss during aging compared to their wildtype (+/+) littermates.

CLAIMS

1. A method for the prophylaxis or treatment of a disease or medical condition which involves excessive bone or cartilage loss comprising administering to a patient an effective amount of an activator of RORα.

2. Use of an activator of RORα in the preparation of a medicament for the treatment of a disease or medical condition which involves excessive bone or cartilage loss.

3. A method for the identification of a compound having bone growth promoting activity which comprises contacting a test compound with a biological system which comprises a functional RORα receptor and which is capable of generating a detectable signal in response to activation of the RORα receptor by a compound having bone growth promoting activity, and monitoring the system for the generation of the signal.

4. A method according to claim 3 for the identification of a compound having bone growth promoting activity comprising contacting the compound with a biological system which comprises a functional RORα receptor and which is capable of generating a detectable signal in response to activation of the RORα receptor by a compound having bone growth promoting activity, measuring the detectable signal in the presence of the test compound and comparing the result obtained with a control.

5. A method according to claim 3 for the comparison of compounds which have bone growth promoting activity, comprising separately contacting the compounds with a biological system which comprises a functional RORα receptor and which is capable of generating a detectable signal in response to activation of the RORα

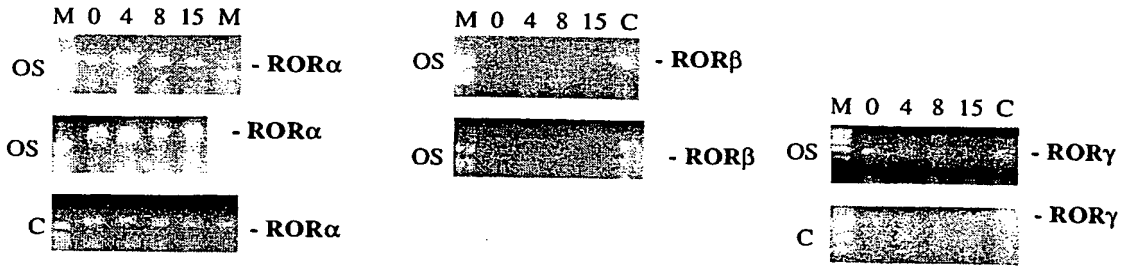
receptor by a compound having bone growth promoting activity, measuring the detectable signal in the presence of each test compound and comparing the signals obtained.

6. A compound when identified by a method according to claim 3.

7. Use of a compound according to claim 6 in a method for the prophylaxis or treatment of a disease or medical condition which involves excessive bone or cartilage loss.

8. Use of a compound according to claim 6 in the preparation of a medicament for the treatment of a disease or medical condition which involves excessive bone or cartilage loss.

Figure 1



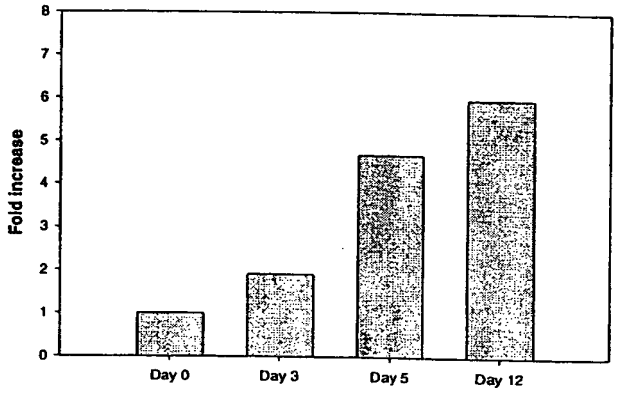


Figure 2

Figure 3

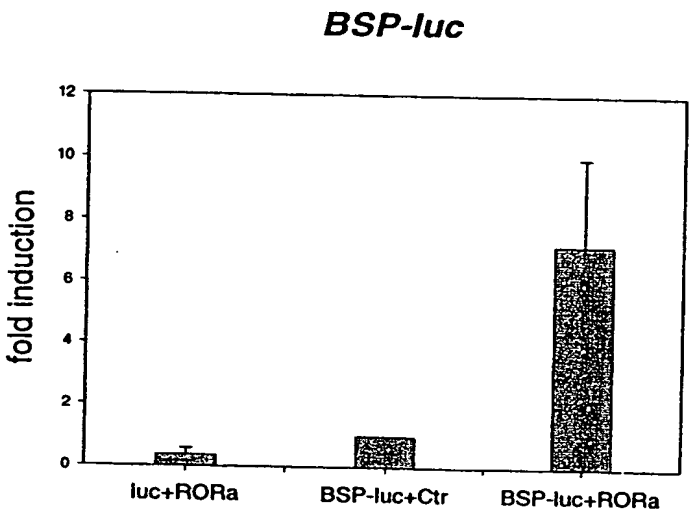
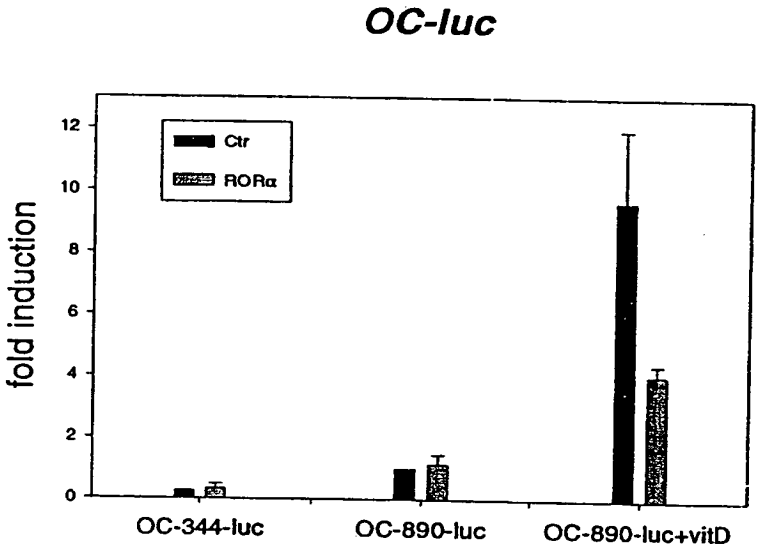
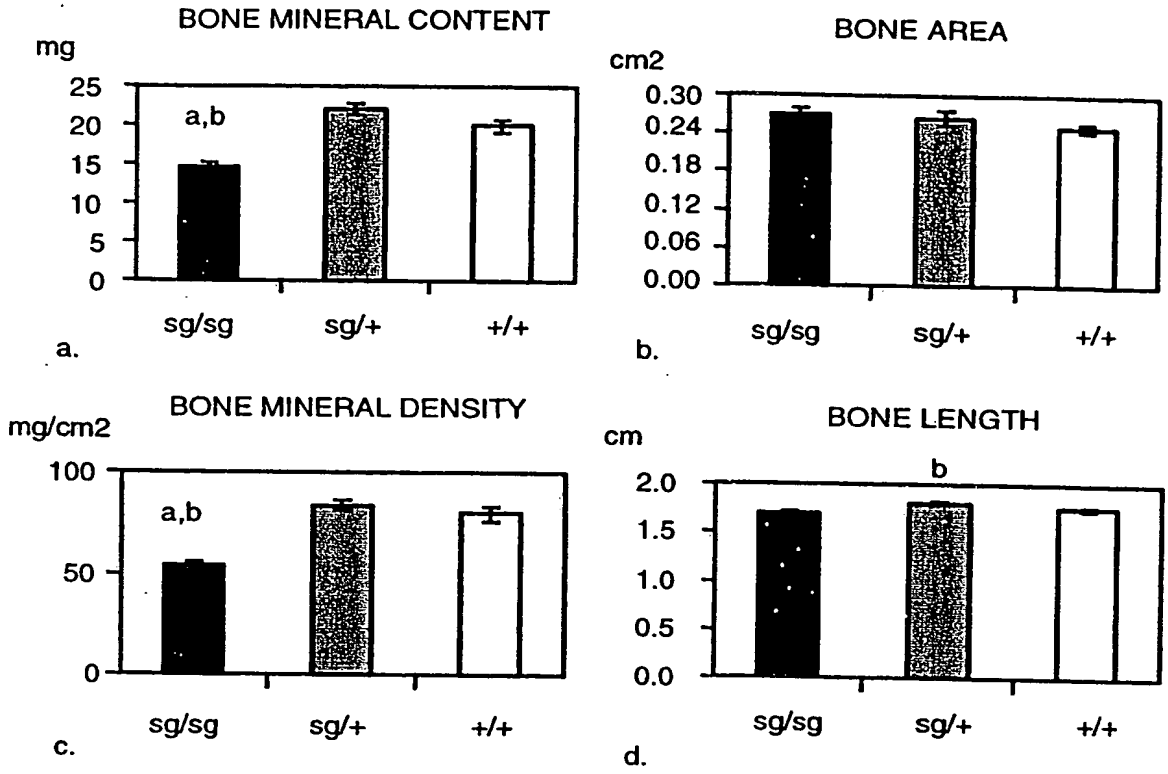

BSP-luc
fold induction
0
2
4
6
8
10
12
luc+RORa
BSP-luc+Ctr
BSP-luc+RORa

Figure 4




BONE MINERAL CONTENT
mg
25
20
15
10
5
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a,b
sg/sg
sg/+
+/+
a.
BONE AREA
cm2
0.30
0.24
0.18
0.12
0.06
0.00
sg/sg
sg/+
+/+
b.
BONE MINERAL DENSITY
mg/cm2
100
50
0
a,b
sg/sg
sg/+
+/+
c.
BONE LENGTH
cm
b
2.0
1.5
1.0
0.5
0.0
sg/sg
sg/+
+/+
d.

Figure 5

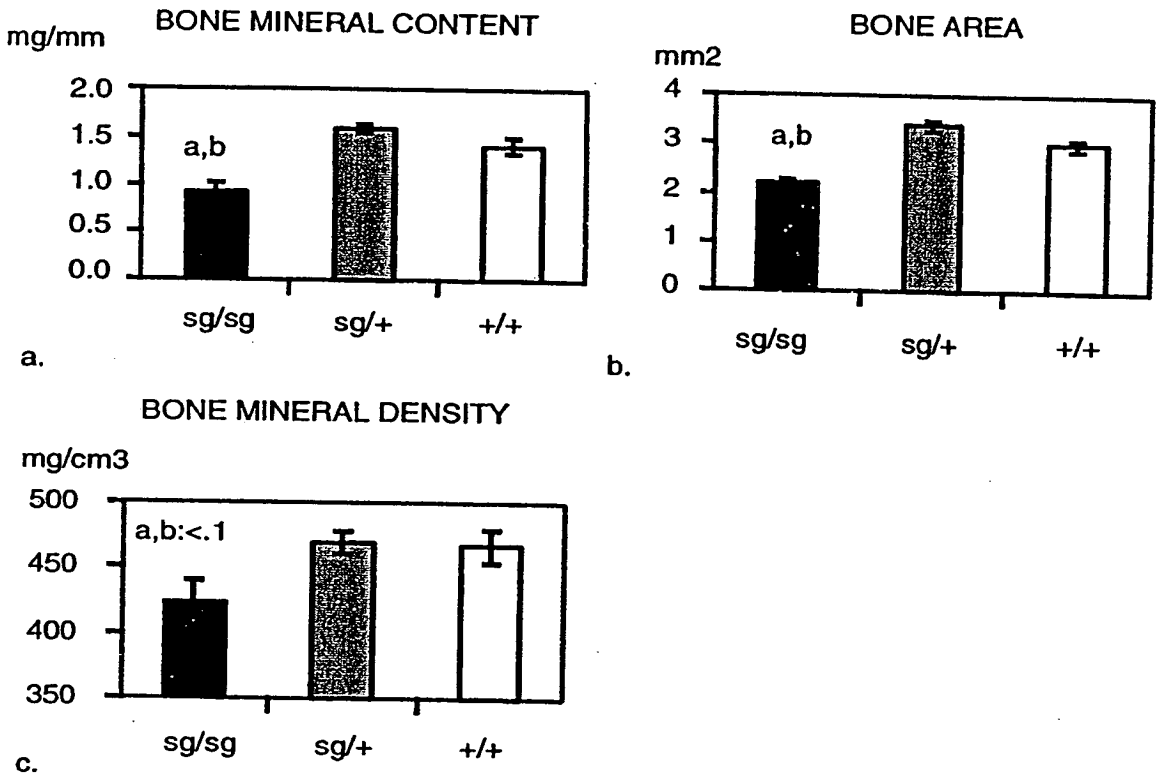

Figure 6

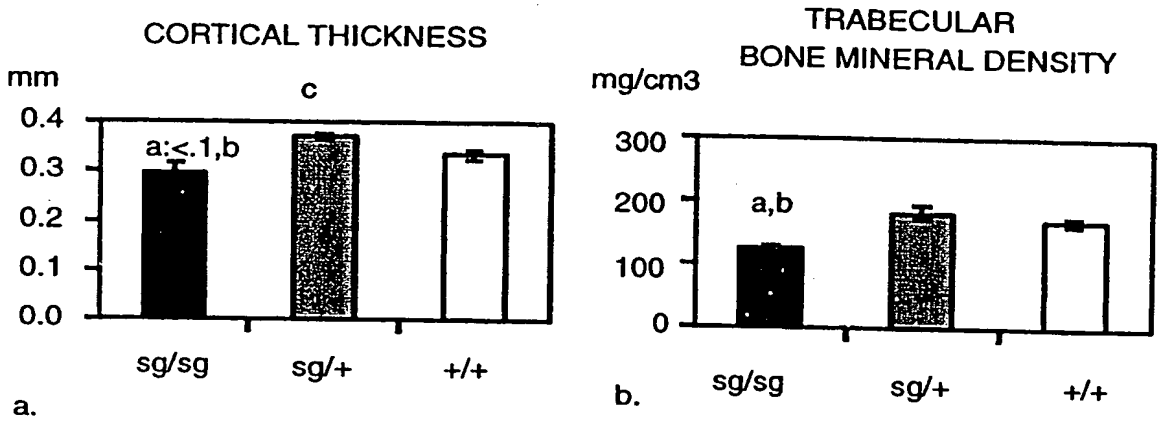
CORTICAL THICKNESS
mm
c
0.4
0.3
0.2
0.1
0.0
a:≤.1,b
sg/sg
sg/+
+/+
a.
TRABECULAR
BONE MINERAL DENSITY
mg/cm3
300
200
100
0
a,b
sg/sg
sg/+
+/+
b.

Figure 7

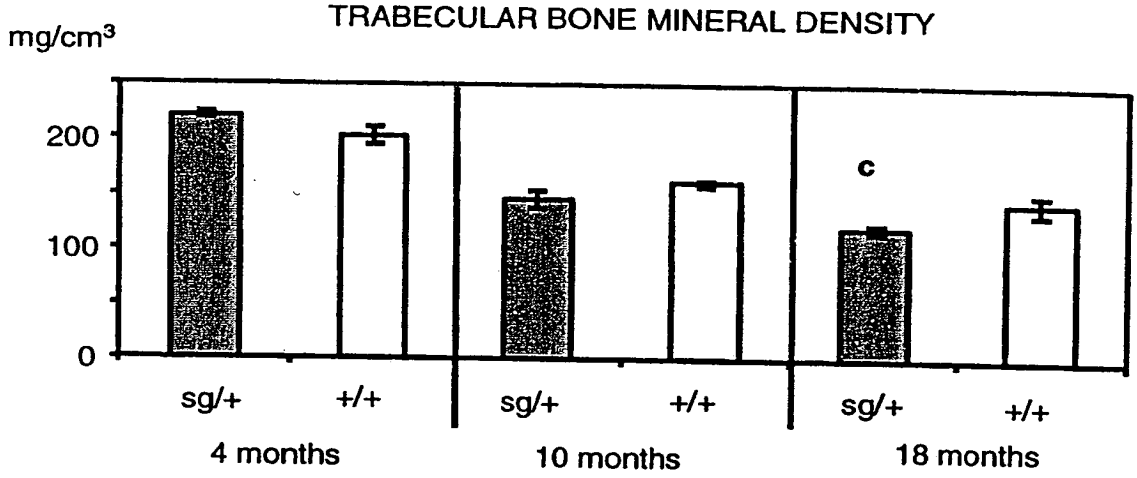
TRABECULAR BONE MINERAL DENSITY
mg/cm³
200
100
0
c
sg/+
+/+
sg/+
+/+
sg/+
+/+
4 months
10 months
18 months

Figure 8

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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18 October 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/26737 A3

(54) Title: TREATMENT OF EXCESSIVE BONE OR CARTILAGE LOSS WITH AN ACTIVATOR OF ROR ALPHA AND ASSAY FOR IDENTIFYING SUCH ACTIVATOR

(57) Abstract: A method for the identification of a compound having bone growth promoting activity comprising contacting a test compound with a biological system which comprises a functional RORα receptor and which is capable of generating a detectable signal in response to activation of the RORα receptor by a compound having bone growth promoting activity, and monitoring the system for the generation of the signal and the use of such compounds for the prophylaxis or treatment of diseases or medical conditions which involve excessive bone or cartilage loss.

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/EP 00/09883

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61P19/02 A61P19/10 A61K45/00 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K G01N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, CHEM ABS Data, SCISEARCH, PASCAL, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 958 683 A (MISSBACH MARTIN ET AL) 28 September 1999 (1999-09-28) cited in the application * col.1, l.66-col.2, l.20; claims 1-23 *	1-7,10
Y	FR 2 776 388 A (LIPHA) 24 September 1999 (1999-09-24) the whole document	1-7,10
Y	EP 0 552 612 A (HOFFMANN LA ROCHE) 28 July 1993 (1993-07-28) * p.2, l.15-26; claims 5-11 *	1-7,10
A	US 5 854 277 A (BENZ GUENTER HANS HERBERT HEIN ET AL) 29 December 1998 (1998-12-29) * col.2, l.12-15 *	1-7,10

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 March 2001	18.04.01

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Authorized officer
Uiber, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 00/09883

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1,2 and 6,7 and 10 relate respectively to a method/use/compound/use defined by reference to a desirable characteristic or property, namely "an activator of ROR alpha" or the use of such an activator.
The claims cover all method/use/compound/use having this characteristic or property, whereas the application provides no explicit support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for such activator. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define said activator by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely the derivatives and teaching taught by US5958683 (=wo9527202) referred to in the description (see p.3, l.1).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr nal Application No
PCT/EP 00/09883

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